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STUDIES OF ZYGOSPORE FORMATION IN PHYCOMYCES NITENS KUNZE

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CYTOLOGICAL STUDIES IN THE MUCORINEAE

II. STUDIES OF ZYGOSPORE FORMATION IN PHYCOMYCES NITENS KUNZE

by

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STUDIES OF ZYGOSPORE FORMATION IN PHYCOMYCES NITENS KUNZE

MARY LUCILLE KEENE

The problem of sexuality in the Mucorineae offers an extremely interesting field of investigation for the physiologist. Great differences have been found to exist with regard to the production of zygospores and sporangia and considerable experimentation has been carried on to determine the conditions influencing asexual and sexual reproduction.

The cytological features are very incompletely known. Numerous studies have been made upon *Sporodinia grandis,* a homothallic form, with more or less conflicting results. They have served, however, to give us some insight into the internal changes which occur when zygospore formation takes place. With the heterothallic forms, on the other hand, there has been little done and the field open here offers many interesting possibilities.

Conjugation in the Mucorineae was first described by Ehrenberg (1820) in a form he termed *Syzygites megalocarpus (Sporodinia grandis).* De Bary (1864) described the process of conjugation and zygospore formation in both *Sporodinia grandis* and *Rhizopus nigricans.* Following this work of de Bary, various workers undertook to study and explain the conditions responsible for the production of zygospores.

Van Tieghem (1876) believed the impoverishment of the medium to be responsible for the production of the zygospores. Cornu (1876) attributed it to desiccation. Brefeld (1872) in an early paper cited cold as an agent but later (1900) he decided that zygospore formation is undoubtedly due primarily to some unknown internal cause. Bainier (1883 *a* and *b,* 1884)

concluded that the production of zygospores is influenced by the medium on which they are grown. Zopf (1888) attributed their formation to the invasion of parasites which caused a decrease in the number of sporangia produced. Klebs (1898, 1902) attributed the immediate cause of sporangial formation to transpiration, the stimulus for zygospore production being concerned with decreased transpiration. Falck (1901) claimed that zygospores would form only when the concentration of the medium is high and that the relative humidity is of slight, if any, importance.

Blakeslee (1904) confirmed Klebs' results showing that increased moisture favors the formation of zygospores. He also showed that the concentration of the medium is unimportant. Blakeslee came to the conclusion, however, that these various physical factors were but secondary influences and that pure sexual strains existed upon which zygospore production was primarily dependent. He obtained cultures of *Rhizopus nigricans* from mycelia which had developed from the suspensors of a zygospore and succeeded in isolating two pure strains which, when grown alone through numerous generations, gave rise to sporangial growth only. If they were "contrasted," that is, grown side by side, or in mixed cultures, they readily produced zygospores. Thus the heterothallic nature of *Rhizopus nigricans* was established. Later he extended his work to include *Absidia caerulea, Phycomyces nitens, Mucor mucedo,* and several undetermined *Mucors,* among the heterothallic forms.

Blakeslee's results have not been extensively tested by other investigators. Hamaker (1906) claimed that zygospores of *Rhizopus nigricans* could be produced unfailingly upon proper media. Namyslowski (1906) also believed that the nutrients were responsible for zygospore production.

The writer (1914) in an earlier paper reviewed rather completely the literature relevant to the cytology of conjugation in the Mucorineae.

The results of studies upon *Sporodinia grandis* published by the writer (1914) vary but little from those published by Dangeard (1906) in his later paper. Fusions in pairs appear to take place between the nuclei of the fusing gametes. Certain plastid-like bodies apparently concerned with the produc-

tion of an oily reserve substance are described. These bodies are small and numerous in partially mature zygospores but later become reduced to one or two large plasmatic bodies saturated with oil. A nuclear disorganization takes place. All of the nuclei do not disorganize and, even in the mature zygospore, many of the normal but slightly larger nuclei are present. The mature zygospore contains a large amount of an irregularly knotted or densely granular, red-staining substance which the writer was unable to explain. It reacted to the triple stain much as the mucorin crystals do, but appeared to arise from the disorganized nuclei.

Burgeff (1915) describes briefly the internal changes which take place at the time of conjugation and of the formation and germination of the zygospores of *Phycomyces nitens*. At the time of conjugation the nuclei become collected at the places of contact of the gametes, a large vacuole becomes evident, the wall is resorbed and the zygospore is established. The nuclei are equally distributed throughout the zygospore. No nuclear fusions are to be noted. Vacuoles containing oil appear, together with granular masses which are protein-like reserve substances. Later the oil vacuoles flow together into a few large globules. At two to four months after formation, one central oil globule is present. The nuclei lie in the outer layer of the non-granular, weakly staining, hollow, cytoplasmic sphere which surrounds the oil globule. The nuclei are homogeneous, without a membrane and are surrounded by a clear zone.

Burgeff finds that, at the time of germination of the zygospores of one variety, the nuclei are arranged in pairs in the periphery. A varying number of the nuclei appear to fuse in pairs. In another variety the conjugation of the nuclei appears to take place before germination. The oil globules become reduced and the cytoplasm becomes vacuolate. As the germ tube pushes out, the nuclei undergo mitotic division. The single chromatin grain of the nucleus forms simple, small chromosomes. The approximate number, determined by count and estimation, is twenty-four. They appear to separate into groups of twelve and become surrounded by a membrane.

The present piece of work was undertaken in the hope that some further facts concerning these internal phenomena might be offered. Furthermore, in view of the work done by Blakeslee

and of the very interesting field opened up by his explanation of the sexuality of the Mucorineae, it was thought possible that some internal differences might be found which could be correlated with the apparent inherent difference between the two strains, or to bring the problem to the point where some such correlation might be possible when the more general cytological features were known.

The previous work was carried out on *Sporodinia grandis,* a homothallic form, in which it is a very simple matter to obtain zygospores in abundance with the simplest cultural methods. The present work, however, has been done on *Phycomyces nitens,* a heterothallic form, in which certain secondary factors are relevant to zygospore production.

I am indebted for my cultures to Dr. R. A. Harper, who kindly supplied me with zygosporic plates in which the two strains had been contrasted and a line of zygospores had resulted in the characteristic fashion where the mycelia of the two strains had come together. Isolations were made from these plates and the pure plus and minus strains resolved. Mixed cultures were also obtained which produced zygospores profusely.

I am also indebted to Dr. L. O. Kunkel for pure plus and minus cultures of *Phycomyces nitens* which were, I believe, of the same stock as those furnished in plate form by Dr. Harper.

METHODS

Stock cultures of the pure plus and minus strains of *Phycomyces nitens* have been kept on various media. Rice or rye bread has been used for the most part because of the ease of manipulation. Small Erlenmeyer flasks are very satisfactory containers for culture work because there is less surface exposed for evaporation and they are less subject to contamination than the wider-mouthed culture dishes that are ordinarily employed. The cotton plugs may be covered with tin foil so that all the moisture is conserved.

At intervals the strains have been inoculated side by side on the various kinds of media and in almost every case a line of zygospores has been evident by the end of a week. Transfers of individual sporangia have been made, one from each strain, with the same marked results when grown together.

Mixed cultures have been kept growing, and here also the zygospore production has been profuse.

For cytological studies, material ranging from three days to six months in age was fixed. Various fixing reagents were employed: Flemming's solution of various strengths, chrom-acetic, Bouin's, and Gilson's. The younger stages were fixed directly on the agar and small portions of the agar were carried through the various stages of washing, dehydrating, and infiltration with paraffin. Because the young gametophores are much convoluted and stand erect from the surface of the substratum, it is difficult to obtain satisfactory sections.

The same difficulties of technic were encountered here which were met with in *Sporodinia grandis,* but in even a more serious form. The older zygospores are extremely brittle because of the heavy walls and the large amount of reserve material contained within the zygospore. The most satisfactory serial sections were obtained from material that was fixed in Flemming's solution, thoroughly washed and treated with hydrofluoric acid while in 95 per cent alcohol. That the resulting structures are normal and not the result of this treatment is evident from the fact that in material treated in the usual ways, the same structures have been found but it is almost impossible to obtain them in serial form because of their torn condition.

For infiltration with paraffin, both the xylol and the cedar oil methods have been employed with equal success. The cedar oil method is probably better for the older stages.

The younger zygospores were cut into sections of from 5 to 10 microns while the older ones were cut into thicker sections ranging from 10 to 60 microns. Owing to the size of the zygospore inclusions found at maturity, the thick sections were more satisfactory for all but the nuclear studies. For affixing the sections to the slides both the albumen-glycerin and the fish-glue fixatives were used.

The Flemming's triple stain was given the preference although anilin-safranin alone was used in many cases. The nuclei stand out quite clearly in such preparations. Gram's anilin-gentian-violet and iodine stain gave some very beautiful preparations. They closely resemble preparations made with Heidenhain's iron-alum-haematoxylin combination but are very much more quickly made.

Stained preparations of the germinating plus and minus spores were made as follows: the spores were germinated either in weak sugar solution or in agar. Bouillon is to be avoided because of precipitation in the subsequent treatment. The spores, germinated in sugar solution, were flooded onto a slide, which had been previously covered with fish-glue fixative, and allowed to stand. When almost dry, the preparation was covered with the fixing solution, either Flemming's or chrom-acetic. The latter is the more satisfactory because subsequent bleaching is not necessary. After an exposure for ten minutes, the fixing agent was drawn off by means of filter paper and the slide was washed carefully in water. Many of the spores washed off but enough remained to give very satisfactory preparations. It was found best to use alcoholic stains if possible because the less the preparations are washed in water the better they are in the end.

The spores germinated in agar were also fixed and carried through to paraffin in the usual ways. These preparations are not as good because the agar is granular and takes the stain and because in sectioning the germ tubes are cut at various angles.

The pure strains of the sporangial cultures were grown on rye bread in large flat dishes. The sporangia were collected at various stages and fixed as previously described. The studies of the sporangia in fixed material were restricted to the large sporangia which are produced in older cultures.

LIFE HISTORY AND DEVELOPMENT

Under natural conditions *Phycomyces nitens* is found growing most commonly in fresh manure. In the majority of texts and general descriptions it is described as preferring an oily substratum, but there seems to be little evidence to support this statement. It is possible to obtain a luxuriant growth on any of the ordinary media used in the laboratory for such purposes, *e. g.*, bread, rice, sugar, and vegetable agars. It requires considerable moisture and medium temperatures (18° to 28°C.) to produce the best vegetative growth.

The spores are small elliptical bodies with resistant walls. They possess from four to twelve nuclei each, typically eight.

The spores germinate readily in water or various weak sugar solutions and broths. They enlarge somewhat and one or two germ tubes push out (Pl. II, fig. 8). These grow for some distance during which time the number of nuclei present increases. Careful studies have revealed no differences between the spores of the two strains. The germ tube branches and sub-branches forming the hyphal network over and through the substratum. At intervals characteristic enlargements occur and occasionally from these, rhizoid-like branches grow out. In other cases, however, these enlargements appear merely as swellings and what they are functionally remains a question. Internally they appear much as any other portion of the mycelium; sometimes there occurs a clustering of the nuclei in this region. The small sporangia which are formed first and are produced close to the substratum appear to be typical in formation and production of spores. The aerial branches which are destined to form the large sporangia appear yellowish with a blackening toward the base. The yellow color is due to the presence of a large amount of oil, as shown by tests, which exudes in droplets when the sporangiophores are crushed. As the sporangiophores elongate they exhibit an extreme sensitiveness to light, the terminal portions bending toward the source of illumination. If grown in the dark, they elongate to a greater extent than when grown in the light.

The history of the formation of the sporangia and of the origin of the spores has been worked out by Swingle (1903). The young sporangiophores are densely crowded with cytoplasm and nuclei. The tip swells out into a small rounded structure. The contents of this are evenly distributed at first but later a zonation appears with the denser portion of the protoplasm toward the periphery and the vacuolate portion at the center. There occurs a marked streaming of the protoplasm into the sporangium at this stage and the rounded vacuoles of the central portion move out to the periphery. They become filled with stainable contents which appear bluish when the triple stain is used. The nuclei are at first evenly distributed through the outer zone, few occurring in the central region. A layer of vacuoles having stainable contents becomes arranged in a dome-shape in the denser portion. The vacuoles flatten, fuse edge to edge and form a continuous cleft which is filled with the same material that filled the vacuoles.

The spore differentiation begins at this time. The vacuoles lose their rounded form and become angular. As the edges or points of the vacuoles come in contact, they unite, forming clefts in the plasm. Furrows cut into the sporeplasm from the columella. These unite with the clefts formed by the vacuoles and cut the plasm into irregular masses. The spores vary in size and in the number of nuclei. The nuclei remain in the resting stage throughout this process; no cases of nuclear divisions were observed by Swingle.

All the cytoplasm and the nuclei are included in the spores themselves. Between the spores there occurs the intersporal slime which has originated in the vacuoles and which Swingle believes arises as a secretion from the vacuolar membrane. It is homogeneous when the spores are formed, stains a bluish brown with the triple stain, and contains no nuclei or other inclusions.

The columella wall begins to form while the spore cleavage is going on and continues to thicken until the spores are ripe. Thus it has been shown in this form, as well as by Harper (1899) for *Pilobolus* and *Sporodinia,* that the columella wall arises as a dome-shaped structure from the very first and not as a straight wall which later arches up, as has been so erroneously described in many places. The spores finally become invested with a refringent cell wall which is very resistant to stains.

Studies of the plus and minus sporangia were made in order to determine whether or not any morphological differences exist between the two strains. The sporangia of the two strains are structurally alike as far as it has been possible to determine. The young sporangiophores of both show a marked increase in the number of nuclei. The nuclei, to all appearances, are similar. The subsequent changes that take place in the formation of the columella and production of spores proceed alike in the two strains.

The character of the growth of the plus and minus strains is unlike, and when cultures of each are grown side by side it is possible to distinguish between them. The mycelium in the minus strain grows somewhat more slowly and less vigorously and the sporangia appear to be fewer and later in appearance.

Zygospore Production

Van Tieghem and Le Monnier (1873) were the first to report the zygospores of *Phycomyces nitens* and in their drawings indicate it as a homothallic form, as a result of which de Bary placed *Phycomyces* with *Sporodinia.* Van Tieghem's first cultures were obtained from cochineal. He lost these but later obtained others from horse dung and again from cochineal. He failed to obtain zygospores when cultures were made from a single sporangium. Bainier (1883*a*) described the zygospores on horse dung mixed with flax-seed flour or soaked with flour, but he also realized the uncertainty of securing them. Later he found that if, during February or March, he started fresh cultures of horse dung he almost always obtained the zygospores within a short time. Zygospores have been reported in *Phycomyces microsporus* by Van Tieghem (1876) and in *Phycomyces pirrotianus* by Morini (1896).

Until Blakeslee (1904) worked with *Phycomyces nitens,* the zygospores had not been reported in this country except in one case in which Thaxter, according to Blakeslee, described them in rabbit dung obtained from Daytona, Florida.

The general facts of the morphology of conjugation in this form have been described by de Bary (1864), to whose account Blakeslee (1904) has added a few points of interest. The majority of writers in describing the origin of the progametes say that the club-shaped progametes develop from the hyphae, increase in size, come in contract and fuse. Blakeslee, however, believes that the stimulus to development comes from contact; that they are "zygotactic" and that the progametes are normally adherent from the first. Blakeslee has devised an ingenious experiment to determine whether the origin of the progametes is dependent upon contact or upon chemical stimuli. The experiment has been repeated by the writer. A small battery jar was lined with filter paper. Suspended by means of wire in the jar, one and one-fourth inches apart, were two small cheese cloth bags containing rye bread. Blakeslee soaked these with orange juice but the author found moistening well with water to be sufficient. An inch of water was put in the bottom of the jar and paper was tied over the top. This was then sterilized in the autoclave at 8 lbs. for 15 minutes. When cool one bag was inoculated with the plus strain and the other

with the minus strain. The paper cover was replaced, tin foil was spread over the top to reduce evaporation, and the apparatus was set into the ice box where evaporation would be slow and light effects negligible. At the end of six days a line of zygospores had formed midway between the two bread bags. Thus, as Blakeslee has pointed out, any influence upon the origin or direction of growth of the gametophores was not the result of any chemical attraction due to the medium or to contact of the mycelial masses, but must have been confined to the aerial portions and is, in all probability, restricted to the hyphae affected.

Blakeslee describes the more general morphological aspects of conjugation and zygospore formation. In a later paper (1906), he describes the germination of the zygospores of *Phycomyces* as well as of several other forms. Germination is said to take place after a long period of rest and it is difficult to bring the zygospores to germination in the laboratory. In the process of germination the outer wall is ruptured and a germ tube forms which produces a rudimentary mycelium on which are borne sporangia and spores. The spores in turn give rise to the next vegetative generation. Presumably it is at the time of the formation of the spores in the germ sporangia that sex segregation occurs. According to Blakeslee, three kinds of spores may be produced in a single germ sporangium: the plus, the minus and the neutral, which upon germination give rise to the corresponding mycelia.

The effects of external conditions, as secondary influences related to the formation of zygospores in this form, have not been carefully investigated by Blakeslee. He secured zygospores on all the substrata tested, which fact disproves the idea that oily substances are essential. He obtained them sparsely on potato agar but plentifully on potato agar acidulated with orange juice. He finds that the zygospores are produced much less abundantly in the warm oven, 26°–28°C., than at room temperature.

The writer has verified these results. In securing zygospores, the following media have been used successfully: carrots, carrot agar, bean dextrose agar, condensed milk agar, potato agar, potato dextrose agar, prune agar, dextrose bouillon agar acidulated with orange juice, bread (both wheat and

rye) and rice. In every case zygospores have been secured when the medium was inoculated with spores from both strains, while at no time and on none of the above-mentioned media, have zygospores been obtained with the pure strains.

No difficulty whatever was experienced in any inoculations in securing a plentiful production of zygospores when the two strains were inoculated into the same medium. The matter of humidity seems to be a very important secondary condition and the best results are invariably obtained if the cultures are kept in a cool, humid place. As soon as the medium begins to dry out, zygospore production ceases and there occurs a marked increase in vegetative development. Considerable difficulty was experienced at first in bringing the zygospores to maturity, but later it was discovered that moisture conditions were responsible. If Petri dishes were used it was essential that a very heavy layer of agar be supplied, and the softer agars with high moisture content were more satisfactory.

This substantiates the work done by Klebs (1898, 1902) in which he pointed out the relation of transpiration to spore formation in *Sporodinia grandis,* and corroborates the results obtained by Blakeslee (1904) on *Rhizopus nigricans.* There seems to be no evidence in *Phycomyces,* at least, that zygospore formation is affected by the concentration of the medium.

Cytology of the Zygospore

When the two branches from sexually different strains of *Phycomyces nitens* come together there occur a branching and lobing which tend to interlock the hyphae (Pl. I, figs. 1 and 2). Previous to contact it is impossible to tell the gametophoric hyphae from any of the other parts of the mycelium. The nuclear conditions at this stage are the same as in the resting mycelium, as far as it has been possible to determine. There is no evidence of any excessive streaming or increase in the number of nuclei. As the branches increase in length, they become erect, the terminal portions elongate, their inner surfaces lying parallel and appressed (fig. 3). The gametophores at this time are usually characterized by a yellow color which is due to the presence of oil as shown by tests. As the progametes continue

to enlarge (fig. 4), the cytoplasm becomes somewhat vacuolate, lines of streaming may be discerned and there occurs a marked increase in the number of nuclei. Although these stages have been studied with great care, it is not possible to say with certainty that nuclear divisions take place. Certain suggestive conditions are found, as indicated in the accompanying figures (Pl. II, figs. 9, 9*a*, 9*b*), but to any one who has attempted a study of the nuclei of these forms it will be evident why a final decision cannot be rendered. The nuclei are so small that only the most conspicuous changes are apparent. It seems highly probable that nuclear divisions do occur at this time, however, as the increase in the number of nuclei in the progametes is greater than can be readily explained through nuclear migration from adjacent parts. Furthermore, these peculiar nuclear conditions are apparent at only four stages: in the progametes; in the suspensors prior to formation of the appendages; in the young sporangiophores; and in the germinating spores. The accompanying figures (9*a*, 9*b*, 10*a*, 10*b*) illustrate the appearance of the nuclei at this time. The resting nucleus is a small, dense, globular to ovoid body possessing a conspicuous, red-staining granule, probably the nucleolus. The chromatin consists of very small granules scattered through the nuclear cavity. The nuclear membrane is fairly well defined in most of the nuclei. At the time of these apparent divisions, there are present from one to three of these red-staining masses and some of the nuclei appear somewhat elongated. In what is evidently a polar view three of these red-staining bodies can be seen, one of which is usually slightly larger than the other two. In edge view, there are usually two of these bodies apparent, sometimes of equal size but often unequal. As far as it has been possible to determine, the larger of these three bodies is the nucleolus and the other two represent the chromatic masses. If they were all nucleoli they would be expected to be visible at other stages. Furthermore, the granular chromatin which is present in the resting nucleus is not found in the nuclei showing this condition. This would suggest that it had become concentrated into these small, deep-staining masses.

The tips of the gametophores remain in contact throughout, but as the gametophores increase in length they also increase in diameter and the portions back of the tip are gradually forced

apart from each other and round out. At this time, a large central vacuole becomes apparent in each and the cytoplasm at the point of contact of the two gametophores is very dense. This is evident in fresh material and is particularly well brought out in stained preparations (figs. 9 and 10). There are few, if any, small vacuoles and the nuclei are very closely placed.

The time of dissolution of the contiguous walls varies here as in *Sporodinia.* Sometimes the gametes are cut off from the suspensors before the protoplasmic masses come into contact, but in other cases, the intervening wall is resorbed before the new delimiting walls are established. This condition has been checked carefully in living material. Figure 10 shows a section through the gametophores before the formation of the cross walls. This formation, however, is a matter of a very short space of time, both being accomplished within an hour. The formation of the delimiting walls proceeds as it does in *Sporodinia.* The new wall forms first at the surface and closes in gradually in the form of a diaphragm which finally severs the intervening strand of protoplasm. The same papilla-like structures found in *Sporodinia* characterize these walls.

The intervening wall separating the two gametes is usually dissolved first at the center but may be resorbed in several places at once. (fig. 10). The protoplasm of one gamete pushes its way into the protoplasm of the other. Here again, as in *Sporodinia,* the author is not inclined to ascribe any significance to this act as due to a sexual differentiation. It appears rather to occur as the result of turgescence. If the internal pressures are not at an equilibrium, which is hardly to be expected, when the wall weakens at one spot, the gamete possessing the greater osmotic pressure is released and surges into the other. Ultimately, the separating wall disappears and the two gametes appear as one mass (fig. 11). Occasionally fragments of this wall may be discerned in the zygospore.

An interesting and somewhat perplexing condition arises in the appearance of the nuclei at this time. As the young zygospore is delimited and the protoplasm of the two gametes begins to mix, there occurs a characteristic grouping of the nuclei. There appear to be from twelve to sixteen nuclei aggregated in a group (fig. 11). It is impossible to state with certainty that in each group there are nuclei from each gamete, as there

is no possible way of distinguishing between the nuclei of the two branches. In fact the nuclei from the two gametes are apparently similar in size and content. It seems highly probable, however, that nuclei from each gamete are aggregated because nuclei in each group fuse in pairs. This is evidenced by the fact that the nuclei show an increase in size and a decrease in number prior to any process of nuclear disorganization. Prior to fusion, the nucleus is dense and granular and possesses a conspicuous nucleolus. After the fusions take place, the nuclear plasm appears vacuolate and the nucleolus is much larger. As indicated in the figures, within one zygospore (fig. 11*c*) there may be found groups of nuclei in which there occur from twelve to sixteen small nuclei and other groups in which there are five large nuclei and two to four smaller ones. There seems little doubt as to the significance of this condition, since no marked differences in the size of the nuclei are evident at any earlier stage.

There are certain large, round, red-staining bodies found within the zygospore at all stages which must not be confused with the nuclei. They are far more conspicuous than the nuclei and are larger. They take the safranin in the same way that the nucleolus does and have undoubtedly been interpreted by some workers as nuclei. They are usually contained within a clear zone and very often each possesses a small secondary body (fig. 11*d*). Their appearance suggests very strongly the crystalloids and globoids of the higher plants. These bodies are characteristic of many of the Mucorineae and have been described by various workers. The author has been able to demonstrate their occurrence at all stages in the life history of both *Sporodinia grandis* and *Phycomyces nitens*. They undoubtedly constitute a form of reserve substance, probably protein in nature. In addition to these, there also occur red-staining angular crystals (fig. 11*e*).

The new wall surrounding the zygospore is established just previous to the nuclear fusions. It is laid down inside of the old gametophore wall (fig. 11). It is more or less roughened and is probably formed in the same way as in *Sporodinia*, which has been described by Vuillemin (1904).

Following the nuclear fusions, there occur nuclear disorganizations. The cytoplasm of the zygospore becomes finely vacuo-

late. The nuclear disorganizations do not appear to be restricted to any particular region but occur throughout the whole zygospore. Some of the nuclei in each group apparently undergo disintegration. This disorganization appears to be restricted to the smaller nuclei or presumably to those which have failed to fuse. During the process of nuclear disorganization the red-staining nucleolus enlarges, stains unevenly and appears as if being dissolved. The margin takes the stain but the central portion stains faintly and may even appear brownish. As disorganization proceeds, and it does so on a large scale, the resulting masses appear to run together or coalesce forming irregular or knotted masses. Plate II, figure 12*b* and Plate III, figures 14*b*, 14*c* illustrate the various stages in the appearance of this substance. The same substance was evident in the zygospores of *Sporodinia*, but both its origin and fate were not interpreted. Here, however, there seems to be little, if any, doubt on these points. As this substance increases in volume it becomes concentrated into one or more central masses. It is without special structure. While it is alveolar in part, it is of a homogeneous texture and in no way resembles living cytoplasm. The oil that is contained within the zygospore sometimes appears as small droplets in this substance but does not mix with it. When fresh zygospores are crushed, gas bubbles may be seen to escape and disappear. Either of these conditions may explain the alveolar nature of this mass.

When fresh mature zygospores are crushed, the substance in question exudes as a semi-transparent amorphous mass. It is insoluble in water. The oil which is pressed out with it is honey-colored and rounds up into distinct globules which react typically to Sudan III and osmic acid. The oil is soluble in xylol and chloroform. The amorphous material, however, is not affected by these reagents. The alcohol in the Sudan III may cause a slight precipitation but the mass is not stained. When absolute alcohol is added, there remains a semi-transparent substance which becomes brownish upon standing. Since the stained sections indicated that this substance might have its origin in the disorganizing nuclei, an attempt was made to test it for proteins. Because of the small amount of material that was available, only rather crude microscopical

tests could be employed. The zygospores were dissected from their black coats, mounted in water and crushed under a cover-glass. The oil exuded in the form of yellow globules, while the amorphous substance was forced out more slowly and in a more or less irregular mass. Sudan III was then added; sometimes the zygospores were mounted directly in this reagent and then crushed. The oil became a brilliant red while the other substance remained clear. If the Sudan III were followed by chloroform, the oil globules immediately disappeared leaving the amorphous mass behind. The addition of ammonia water caused the latter to disappear at once. Sodium hydrate had much the same effect but acted more slowly. The xantho-proteic test was applied to other crushed zygospores and this mass took on a decided orange color. Millon's reagent failed to give the color changes. Zygospores were crushed in alkaline phenolphthalein and apparently gave an acid reaction, since the portion of the reagent through which the zygospore content was forced lost its color. The significance of this, however, is not great, because the reaction of the rest of the cytoplasm may have been acid. On the other hand, the cytoplasm and nuclei at this time are reduced to a thin parietal layer, as shown in sections (Pl. III, fig. 17), so the acid reaction was probably caused by these amorphous inclusions. Since it was impracticable to attempt to isolate this substance in question, the conclusions based upon these tests can only be of the most tentative nature but the reactions appear to be those of the nucleoproteins. These tests have served merely to separate this type of reserve material from the oil which is present and to aid in the interpretation of a rather perplexing condition existing in the mature zygospore.

At the time that the nuclear disorganizations begin, there appears a marked zonation in the zygospore (Pl. II, fig. 12). The inner coat of the zygospore, which is semi-transparent, is produced at this time. It is very thick and resistant. It is laid down within the brown, roughened wall previously formed. Thus the mature zygospore is invested with two thick coats (Pl. III, fig. 14) and parts of the original gametophore walls may still be found on the outside in many places. Toward the margin of the zygospore there appear bluish-staining areas. In the earlier conditions they appear merely as vacuoles in which

there are stainable contents. When fixed with Flemming's solution these areas are blackened by the osmic acid and require considerable bleaching before they are clear. They are responsible for the zonate appearance of the zygospore at this time (fig. 13).

The zygospore becomes more and more vacuolate, the groups of nuclei become separated, and the disorganizing nuclei may be found distributed throughout the zygospore (Pl. II, fig. 12). As the vacuolate condition increases the center of the zygospore becomes quite sponge-like in appearance. The bluish-staining bodies enlarge and begin to appear more or less reticulate (Pl. III, fig. 15). They are identical with the bodies described by the writer in *Sporodinia grandis* and are undoubtedly associated with the formation of oil. They do not seem to play as prominent a part in the maturation processes in *Phycomyces* as they do in *Sporodinia.* It may be that the protein material just described replaces to some extent, as reserve material in *Phycomyces,* the oil that is so plentiful in *Sporodinia.*

The literature referring to the possible relationship between these structures associated with oil production in the Mucorineae and the elaioplasts described by many workers, has been reviewed by the witer in a previous paper (1914). The elaioplasts or oil plastids, according to these various investigators, are bodies set aside by the protoplasm for the secretion of oil.

In *Sporodinia* the oil plastids arise as very small globular bodies. When first formed they appear as vacuoles with stained contents but careful study of them under high powers of magnification shows a coarse reticulation with a more or less homogeneous center. As they increase in size, their reticulate nature becomes more and more apparent. A coalescence between them takes place, apparently as the result of crowding, and in the mature zygospore there may occur from one to three of these plastids but usually only one large one is present

In *Phycomyces* these reticulated bodies, as already described, appear to originate at various places through the zygospore, being most apparent toward the margin. Oil is associated with them from their earliest appearance. As the zygospore matures these plastid-like bodies become vacuolate, enlarge and assume the appearance of definite cell structures. In the older zygospores studied, numerous small plastids were still to be found toward the periphery, but, as indicated in the accompanying

figures (15 and 16), a single large mass is often found. This plastid in *Phycomyces* does not resemble very closely the large plastid found in the mature zygospores of *Sporodinia,* but it undoubtedly functions in the same way. In *Sporodinia* the large plastid is finely granular and appears delicately reticulate. In *Phycomyces* its protoplasmic nature is not as evident. It is coarsely reticulate and even alveolate. It appears as if the protoplasm, when once saturated with oil, loses its granular appearance and may even deliquesce.

The large protein masses do not resemble the oil plastids either in color or in consistency in the prepared sections. In figure 16, the disorganized nuclei have not as yet become coalesced as they do somewhat later, as shown in figure 17. The amount of oil apparently becomes somewhat reduced in the older zygospores. The proportional amount of each substance varies in different zygospores.

Nuclei of the original typical form can be found throughout all the different stages described above. They stain somewhat differently, depending on the age of the zygospores. They are much reduced in number in the older zygospores but are still plentiful enough to be evident in many places.

There are several points which emphasize the fact that nuclear disorganizations take place. The one just mentioned, namely, that there is a marked reduction in the number of nuclei present in the mature zygospore, is the most important; secondly, the nuclear disorganization exactly parallels in appearance the conditions found in the suspensors, where there is no question concerning the occurrence of both nuclear and cytoplasmic disintegration. Swingle (1903) has described nuclear disorganizations in the sporangia of both *Rhizopus nigricans* and *Phycomyces nitens* as follows: *The first sign of disintegration is the appearance of a red-staining mass on one side. As the process goes on, the whole nucleus comes to appear as a slightly shrunken, homogeneous mass, often irregular in shape, and staining the same shade of red as the crystalloids. It might be argued that these red-staining bodies are crystalloids whose substance is being dissolved but I have very good evidence that such is not the case. * * * There are all stages of disintegration between the almost perfect nuclei and the most shrunken ones.*

Aside from these facts, on any conception of a nuclear-

cytoplasmic balance, it seems improbable that the immense number of nuclei which are present in the zygospore following the fusion of the two gametes, should persist in such a comparatively small structure, and nuclear disorganizations might normally be expected to occur.

There occurs an interesting change in the development which is of importance from a secondary standpoint only. Following the formation of the delimiting walls in the gametophores, there appear slight protuberances from the walls of the suspensors (Pl. I, fig. 6). These grow rather rapidly and give rise to the peculiar, dichotomously-branched appendages which grow out and surround the zygospore giving to it a very characteristic appearance (fig. 7). These appendages first appear on the same suspensor in which septation first occurred. This, however, does not appear to be due to any inherent difference between the two strains. Internally the changes are interesting.

Nuclear divisions appear to follow the cutting off of the terminal portions, or the nuclear divisions inaugurated in the progametes continue, and there results an increase in the number of nuclei in the suspensors. In a cross section of the suspensors there appear peculiar deep-staining areas opposite the places from which the appendages grow. The nuclei in these areas appear as if caught in a current and even show elongation in the direction of the current. The appendages as they elongate become filled with protoplasm and later appear vacuolate with only a thin lining layer of protoplasm in which the nuclei are distributed in about the same proportion as in the vegetative mycelium. All the protoplasm and nuclei disappear in the older stages and the appendages and suspensors appear empty (Pl. III, fig. 14). Occasionally dense, red-staining bodies may be found in the suspensors. They resemble closely the protein masses found in the partly mature zygospore and are probably of similar origin.

Zygospore germinations were secured in only one small lot of material so it has not been possible to carry the history of the internal changes to completion. It will be interesting to know what is the fate of these various substances in germination and what are the nuclear behaviors which complete the cycle.

Thus it will be seen that fundamentally, the changes which take place when sexual reproduction occurs in *Phycomyces*

nitens are essentially the same as those that occur in *Sporodinia grandis.* The conditions of nuclear fusions and subsequent disintegrations are the same. There appear, in both forms, certain plastid-like bodies which are associated with the oil which occurs as a reserve substance. In *Sporodinia* the disintegration of the nuclei results in the formation of an irregular knotted mass of material which in *Phycomyces* appears as a mass of what is probably either nucleic acid or nucleo-protein substance.

Careful cytological studies of the plus and minus strains of germinating spores, young sporangiophores, sporangia and progametes have revealed nothing in the nature of a morphological sexual differentiation. The author is satisfied that if any such means of differentiation between the two strains is to be found, it must be looked for in the germ sporangia where segregation undoubtedly occurs or more likely in physiological conditions. Perhaps microchemical tests will reveal a difference in the materials present in the two strains, especially in the progametes.

Many workers have described coenocentra in the Phycomycetes and McCormick (1912) has described the presence of a coenocentrum in the zygospores of *Rhizopus nigricans* but in *Phycomyces nitens* nothing has been encountered which answers the descriptions of a coenocentrum. While aggregations of nuclei occur, they are entirely independent of any coenocentra-like structures.

Young and partially mature zygospores have been studied in *Rhizopus nigricans,* another heterothallic form, and much the same set of conditions has been found as described in the present paper. The nuclei show the same clustered condition and subsequent nuclear disintegrations occur. The nature of the reserve substances in the mature zygospores has not been determined as yet. Work on *Zygorhynchus moelleri* was abandoned temporarily because of the very small size of the zygospores and the accompanying difficulties of technic. But, in view of the present work, there remains open an interesting problem in connection with such forms as show a morphological differentiation between the two sexual branches. It is interesting to note, as Blakeslee has pointed out, that in the heterothallic forms where we should expect to find an inequality of the gametes none occurs uniformly, while in *Zygorhynchus* and

Dicranophora, homothallic forms, the morphological differentiation is marked and constant.

Summary

1. There are no morphological differences to be found in the germinating spores of the plus and minus strains of *Phycomyces nitens.* They both germinate by means of one or two germ tubes which branch and produce the vegetative myceilum.

2. The vegetative mycelium of the minus strain grows somewhat less vigorously than that of the plus strain and the sporangia appear later.

3. The sporangia of the plus and minus strains are similar in all respects.

4. The two strains—the plus and the minus—are necessary in order to secure zygospores.

5. Zygospores were secured on various kinds of media and their production does not seem to be in any way dependent upon the concentration of the medium. Humidity, however, appears to be an important secondary factor.

6. Interlocking of lobes occurs when certain hyphal branches of the plus and minus strains come in contact. This appears to be the result of contact and not of chemical stimulation.

7. The terminal portions of these branches elongate forming the progametes. There is no difference morphologically between the two progametes.

8. The progametes show an increase in the number of nuclei as they increase in size and round out from each other. This increase in the number of nuclei is undoubtedly due to nuclear divisions.

9. Delimiting walls are formed which cut off the terminal portions, resorption of the contiguous walls occurs, the two gametes come in contact, and a mixing of the protoplasm takes place.

10. Grouping of the nuclei follows. There occur from 12 to 16 nuclei in each group.

11. Nuclear fusions in pairs follow, resulting in the reduction in the number of nuclei and in an increase in the size of the nuclei.

12. Deep-staining, crystal-like bodies are to be found at all stages. They resemble the crystalloids and globoids of the higher plants and undoubtedly constitute a form of reserve substance.

13. Zonations within the zygospore take place at the time of the formation of the second wall which invests the zygospore, and at the time of the appearance of the oil masses.

14. Disorganization of some of the nuclei follows and results in the formation of a large amount of irregular to globular masses of a deep-staining substance.

15. The oil plastids enlarge, show reduction in number and appear as definite reticulate bodies.

16. In the mature zygospore there are found two different types of reserve material.

a. A large amorphous mass of what is probably nucleoprotein substance.
b. A considerable amount of oil.

17. Nuclei of the typical form are found in much reduced numbers in zygospores six months old. The protoplasm is reduced to a thin parietal layer.

This work has been done under the direction of Professor G. M. Reed of the University of Missouri and I am greatly indebted to him for his unfailing interest and valuable suggestions.

Madison, Wisconsin.

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EXPLANATION OF FIGURES IN PLATES.

All figures were drawn with the aid of the camera lucida and with the Zeiss apochromatic objectives and compensating oculars. Figures 8*a*; 9*a*, *b*, *c*; 10*a*, *b*; 11*a*, *b*, *c*, *d*, *e*; 12*a*, *b*; 14*a*, *b*, *c*; 17*a*, *b*, were drawn with objective 1.5 mm. and ocular 12. Figure 8 was drawn with objective 1.5 mm. and ocular 8. Objective 16 mm. and ocular 12 were used with the remaining figures.

Plate I.

Figs 1 and 2. Young gametophores of plus and minus strains showing lobing and interlocking.

Fig. 3. Young gametophores showing elongation of terminal portions. It is at this stage that the gametophores appear as erect yellow columns.

Fig. 4. Enlargement of gametophores.

Fig. 5. Formation of the delimiting walls.

Fig. 6. Showing the origin of the spine-like appendages.

Fig. 7. Mature zygospore with blackened wall and invested with appendages.

Plate II.

Fig. 8. Germinating spore of plus strain.

Fig. 8*a*. Nuclei from germinating spore under higher magnification.

Fig. 9. Section through progametes prior to septation or resorption.

Figs. 9*a* and *b*. Nuclei from respective progametes. These may illustrate phases of nuclear divisions.

Fig. 9*c*. Crystalloids.

Fig. 10. Young conjugating branches at the time of resorption of the separating wall.

Figs. 10*a* and *b*. Nuclei from respective suspensors.

Fig. 11. Young zygospore, showing characteristic grouping of the nuclei following fusion of the protoplasmic masses.

Fig. 11*a* and *b*. Nuclei of the suspensors at time of formation of appendages.

Fig. 11*c*. Groups of nuclei showing conditions before and after nuclear fusions.

Figs. 11*d* and *e*. Crystalloids.

Fig. 12. Zonation at time of formation of second zygospore wall.

Fig. 12*a*. Nuclei at this stage.

Fig. 12*b*. Disorganizing nuclei.

Plate III.

Fig. 13. Zonation at time of formation of oil bodies.

Fig. 14. Showing disorganizing nuclei and aggregation of irregular masses resulting. Shows also the two walls of the zygospore and a small portion of the old gametophore wall.

Figs. 14*a*, *b*. Appearance of nuclei and disorganizing nuclei at this stage.

Fig. 14*c*. Irregular masses formed by coalescence of disorganizing nuclei.

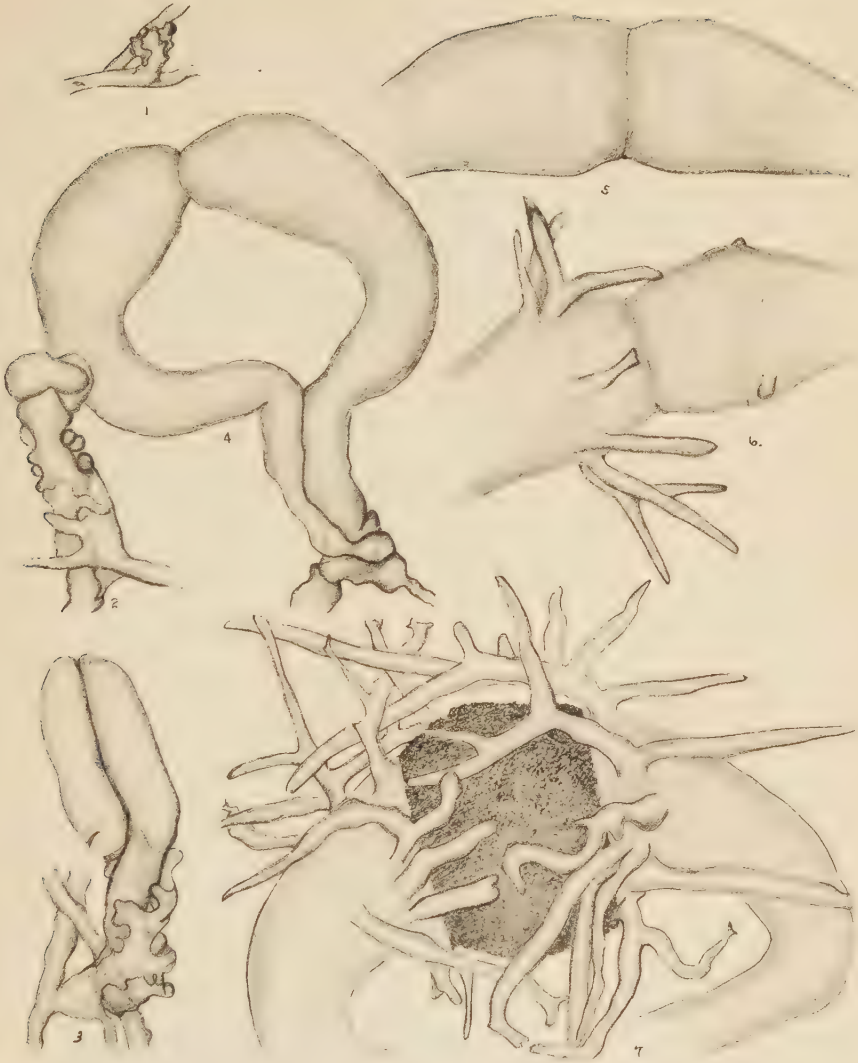
Fig. 15. Appearance of reticulated oil plastids.

Fig. 16. Large oil plastid and deep-staining area of disorganizing nuclei.

Fig. 17. Mature zygospore showing large alveolated nucleo-protein mass which results from the disorganized nuclei.

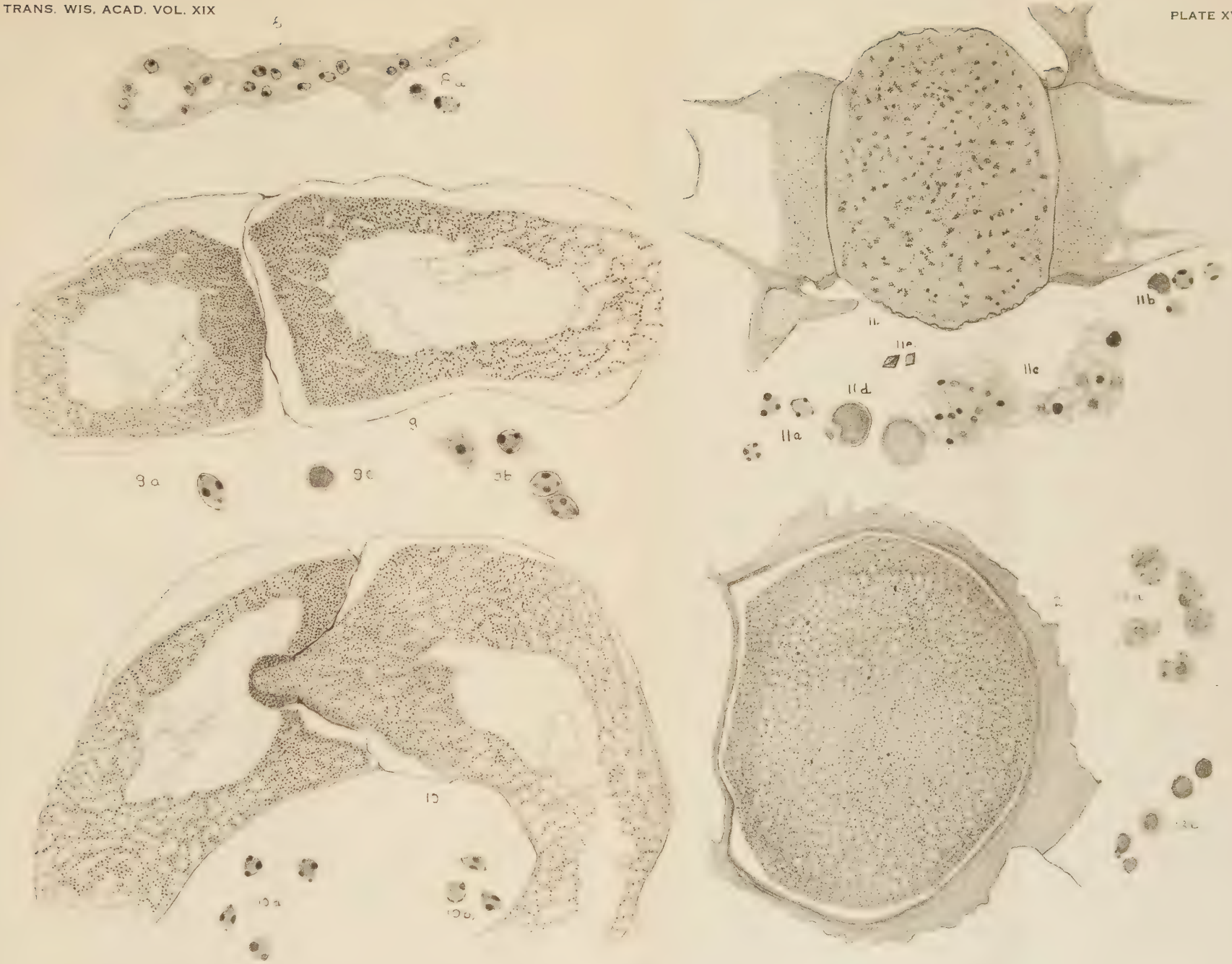
Fig. 17*a*. Appearance of a portion of the nucleo-protein mass under higher magnification.

Fig. 17*b*. Nuclei of the mature zygospore, much reduced in number.



KEENE—ZYGOSPORE FORMATION IN PHYCOMYCETES

COCKAYNE BOSTON



KEENE—ZYGOSPORE FORMATION IN PHYCOMYCETES

COCKAYNE, BOSTON

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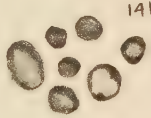

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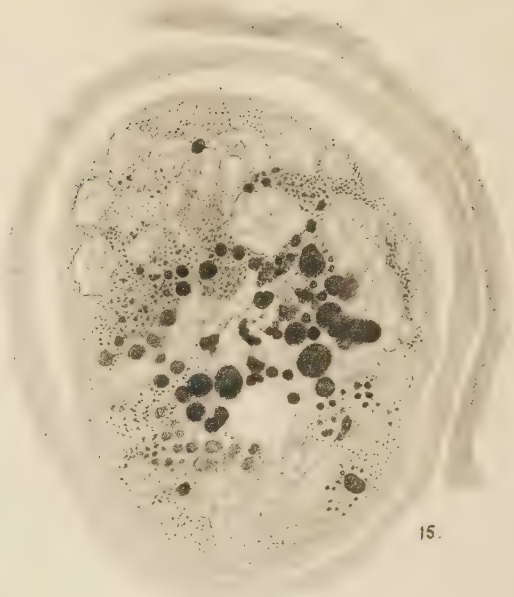

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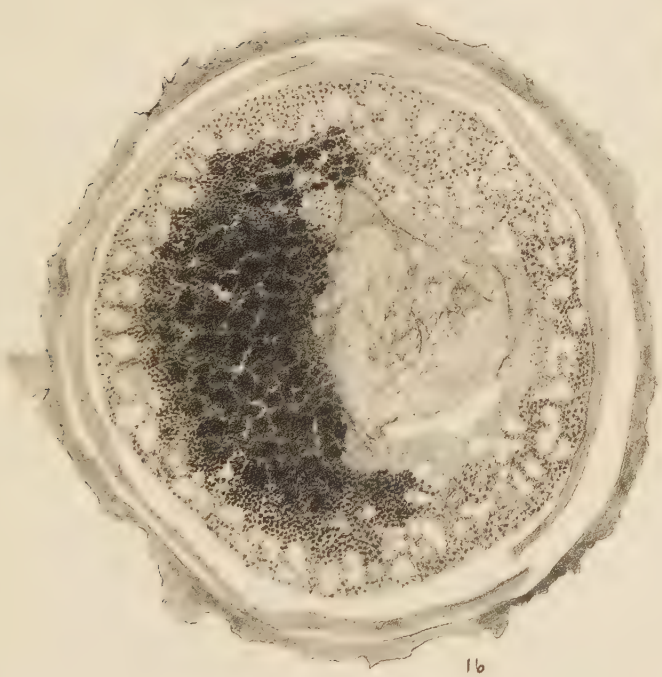

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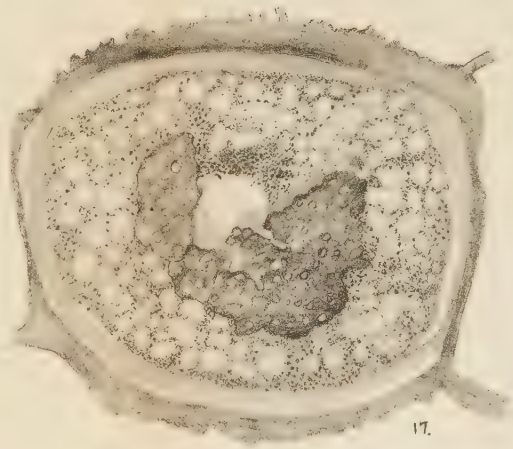

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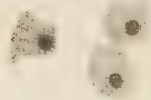

KEENE—ZYGOSPORE FORMATION IN PHYCOMYCETES

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